THE ENZYMATIC DEAMINATION OF AMPHETAMINE (BENZEDRINE)

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A previous study on the metabolism of amphetamine (1-phenyl-2-aminopropane), widely used as a stimulant of the central nervous system, showed that its major route of biotransformation in the rat and dog involved hydroxylation of the aromatic ring. In the rabbit, however, the compound was not hydroxylated, but was found to be transformed in another manner (2).

The present report describes an enzyme system in the rabbit which deaminates amphetamine to yield phenylacetone. It will be shown that the enzyme system is localized in the microsomal fraction of the cell and requires oxygen and reduced triphosphopyridine nucleotide. Furthermore, these studies indicate that there are inhibitory factors present in the liver of the dog, rat, and guinea pig which may explain, in part, the inability of these species to deaminate amphetamine.

Materials—*l*- and *d*-amphetamine sulfate was obtained through the courtesy of the Smith, Kline and French Laboratories. *l*-Norephedrine hydrochloride was kindly supplied by K. H. Beyer of Sharpe and Dohme. Triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) 80 per cent purity, glucose-6-phosphate dehydrogenase, and glucose-6-phosphate were obtained from the Sigma Chemical Company. Reduced triphosphopyridine nucleotide (TPNH) was prepared by the procedure of Kaplan *et al.* (3).

Methods—Amphetamine, ephedrine, norephedrine, and methylamphetamine were determined by the methyl orange reaction of Brodie and Udenfriend (4) with benzene as the extractant, as previously described (2, 5). An essentially similar procedure was used for the estimation of 1-phenyl-1-aminopropane, 1-phenyl-3-aminobutane, phenylethylamine, isoamylamine, 2-aminoheptane, and benzylamine with chloroform as the extractant. Tyramine was estimated according to Udenfriend and Cooper (6) and *p*-

* A preliminary report of this work has appeared (1). The material in this investigation will appear in a thesis in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology, Graduate Council, George Washington University.

hydroxyamphetamine according to Axelrod (2). Ammonia was estimated by the Conway microdiffusion procedure (7).

Phenylacetone was determined by its reaction with 2,4-dinitrophenylhydrazine as follows: An aliquot of biological material was transferred to a 40 ml. glass-stoppered centrifuge tube containing 0.5 ml. of 1 N NaOH and 20 ml. of carbon tetrachloride (reagent grade) and shaken for 20 minutes. The tube was centrifuged and the aqueous phase removed by aspiration. The carbon tetrachloride extract was washed with 5 ml. of water to remove "blank" material which reacted with dinitrophenylhydrazine. 15 ml. of the carbon tetrachloride phase were transferred to a 40 ml. glass-stoppered centrifuge tube containing 5 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl and shaken for 2 hours. The aqueous phase was removed by aspiration and the organic phase washed three times with 5 ml. portions of 2 N HCl to remove unchanged dinitrophenylhydrazine. 6 ml. of the carbon tetrachloride extract containing the dinitrophenylhydrazone derivative of phenylacetone were transferred to a cuvette containing 1 ml. of 10 per cent KOH in ethanol, and the optical density was read in a spectrophotometer at 430 mμ. A blank consisting of biological material carried through the procedure was set at 100 per cent transmission. Standards were prepared by adding a known amount of phenylacetone to biological material and proceeding as above. Phenylacetone added to biological materials in amounts from 0.5 to 5 μmoles are recovered with adequate precision 90 ± 6 per cent.

Preparation of Tissue Samples—The preparation of all tissue samples was carried out at 0–3°.

Male albino rabbits were stunned and exsanguinated. The livers were immediately removed and homogenized with 2 volumes of 0.1 M phosphate buffer, pH 7, with a Potter-Elvehjem type of homogenizer. The homogenates were centrifuged at 9000 × *g* for 10 minutes to remove unbroken cells, nuclei, and mitochondria, and the resulting supernatant fraction was dialyzed against 0.01 M phosphate buffer, pH 7.0, for 20 hours. The supernatant fraction could be stored for at least 2 weeks at −10° without loss of activity.

The particulate fractions of liver were prepared by differential centrifugation of 12 per cent homogenates in 0.25 M sucrose (8). The nuclear fraction was sedimented by centrifugation at 600 × *g* for 10 minutes; the mitochondria at 9000 × *g* for 10 minutes; microsomes were separated from the soluble supernatant fraction by centrifugation at 78,000 × *g* for 45 minutes. The microsomes were washed once with 0.25 M sucrose and recentrifuged. Particulate fractions were resuspended in a 0.1 M phosphate buffer, pH 7.0, to a concentration 3 times that of the original homogenate. The particulate and soluble supernatant fractions were dialyzed against 0.01 M phosphate buffer, pH 7.0, for 20 hours.

Measurement of Enzyme Activity—A typical incubation mixture was prepared as follows: In a 20 ml. beaker were placed 0.3 ml. of enzyme preparation (equivalent to 100 mg. of liver), 5 μmoles of nicotinamide, 0.1 μmole of TPN, 5 μmoles of $MgCl_2$, 0.6 μmole of *l*-amphetamine, 0.5 ml. of phosphate buffer pH 7.4 (0.2 M), and water to make a final volume of 4 ml. The mixture was incubated in a Dubnoff metabolic shaking apparatus for 2 hours at 37° in air. At the end of the incubation period an aliquot of the reaction mixture was immediately transferred to a 60 ml. glass-stoppered bottle containing 0.5 ml. of 1 N NaOH and assayed for amphetamine. Enzyme activity was expressed as micromoles of amphetamine metabolized.

After the incubation the residual amphetamine was identified by comparing its distribution ratios between buffers of various pH values and benzene with those of authentic amphetamine. The results indicated that the apparent amphetamine measured after incubation had the same solubility characteristics as authentic amphetamine and that the compounds were presumably the same.

Results

Tissue Distribution of Enzyme Activity—*l*-Amphetamine (0.6 μmole) was incubated with 100 mg. of minced liver, lung, diaphragm, muscle, kidney, and brain of the rabbit under the conditions described under "Methods." The liver metabolized 0.35 μmole of *l*-amphetamine in 2 hours, while the other tissues were unable to metabolize the drug.

Properties of Enzyme System—An undialyzed supernatant fraction of rabbit liver failed to metabolize *l*-amphetamine in appreciable amounts without the addition of nicotinamide and either TPN or DPN. Nicotinamide presumably served to protect the pyridine nucleotides against enzymatic destruction (9). The effect of the two pyridine nucleotides was then examined in a supernatant fraction of rabbit liver dialyzed for 20 hours. It was found that the activity of the dialyzed supernatant fraction was restored after the addition of TPN, while DPN was inactive (Fig. 1). Mg^{++} stimulated activity about 10 per cent. Negligible activity was observed under anaerobic conditions, but incubation in air was as effective as incubation in oxygen. In phosphate buffers the maximal enzyme activity occurred at pH 7.4, and the activity was sharply reduced below and above this pH.

Rate of Metabolism—The rate of metabolism of *l*-amphetamine by the rabbit supernatant fraction is shown in Fig. 2. About 50 per cent of the drug was metabolized in 3 hours, with no further metabolism after this time. To examine the lability of the enzyme, the rabbit liver supernatant fraction was preincubated at 37° for 3 hours. On the addition of *l*-amphetamine, no metabolism of the drug took place.

The relationship between substrate concentration and substrate disappearance is shown in Table I. At concentrations of amphetamine varying from 0.6 to 2.4 μmoles a constant amount of the drug was metabolized.

Intracellular Localization of Enzyme System—Nuclear, mitochondrial, microsomal, and soluble supernatant fractions of the rabbit liver were separated by differential centrifugation (8) and assayed for their capacity to metabolize *l*-amphetamine. Negligible enzyme activity was observed in each cellular fraction (Table II). However, when the microsomal and soluble supernatant fractions were combined, the drug was metabolized

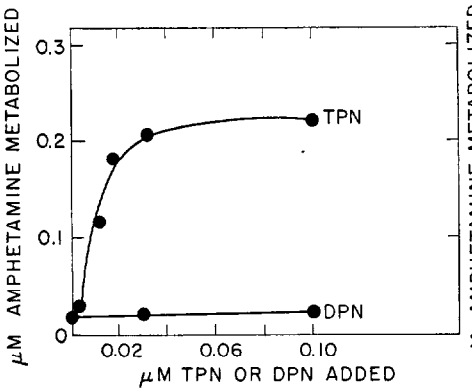


FIG. 1

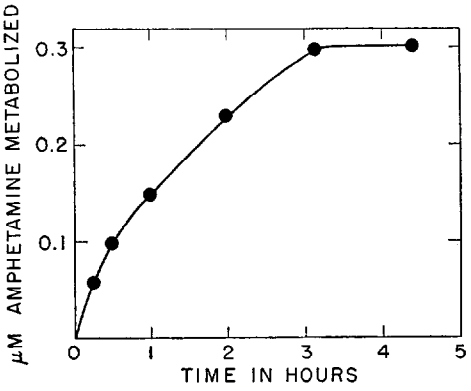


FIG. 2

FIG. 1. Requirement for TPN. 0.3 ml. of dialyzed rabbit liver supernatant fraction was incubated for 2 hours at 37° with 0.6 μmole of *l*-amphetamine, varying amounts of TPN or DPN, and cofactors described under "Methods."

FIG. 2. Rate of metabolism of *l*-amphetamine. Six beakers, each containing 0.3 ml. of rabbit liver supernatant fraction, were incubated at 37° with 0.6 μmole of *l*-amphetamine and cofactors described under "Methods."

almost as effectively as by the whole homogenate. From these observations it was evident that factors present in both the microsomes and the soluble supernatant fraction were required to carry out the metabolism of amphetamine.

Requirement for TPNH—The possible rôle of the soluble supernatant fraction in the metabolism of amphetamine was examined by adding a number of substrates normally present in this fraction to the microsomes. It was found that by replacing the soluble supernatant fluid with TPN and either glucose-6-phosphate or isocitric acid, unwashed microsomes metabolized amphetamine; washed microsomes in the presence of these factors were unable to metabolize the drug. These observations suggested that TPN-dependent dehydrogenases associated with the unwashed microsomes were involved in the enzymatic conversion of amphetamine. In

the presence of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and TPN, washed microsomes metabolized amphetamine (Table III). These results suggested that reduced TPN was the actual cofactor and that

TABLE I

Relationship between Substrate Concentration and Substrate Disappearance

0.3 ml. of rabbit liver supernatant fraction was incubated at 37° for 2 hours with various amounts of *l*-amphetamine and cofactors described under "Methods."

l-Amphetamine added	*l*-Amphetamine metabolized
μmoles	*μmole*
0.12	0.12
0.30	0.20
0.60	0.25
1.20	0.24
2.40	0.22

TABLE II

Intracellular Distribution of Amphetamine Deamination Activity

Various intracellular fractions were prepared from rabbit liver by differential centrifugation as described under "Methods." Aliquots of the various fractions equivalent to that contained in 100 mg. of whole rabbit liver were incubated at 37° for 2 hours with 0.6 μmole of *l*-amphetamine and cofactors described under "Methods."

Intracellular fraction	*l*-Amphetamine metabolized
	μmole
Whole homogenate	0.27
Nuclei*	0.06
Mitochondria	0.00
Microsomes	0.03
Soluble supernatant	0.00
Nuclei* and soluble supernatant	0.08
Mitochondria and soluble supernatant	0.02
Microsomes and soluble supernatant	0.20

* This fraction contained unbroken cells and red blood cells as well as nuclei.

TPN-dependent dehydrogenases in the soluble fraction of the cell served to generate this cofactor. To confirm this, the effect of chemically prepared TPNH on the metabolism of amphetamine by microsomes was examined. Reduced TPN was found to be as effective as the supernatant fraction in promoting the metabolism of amphetamine by the washed microsomal fraction of rabbit liver (Table III). DPNH could not replace TPNH. From these results, it appears that the supernatant fraction

served to maintain a reservoir of reduced TPN by catalyzing the oxidation of glucose-6-phosphate and other substrates and that the deamination of amphetamine was mediated by a TPNH-dependent enzyme located in the microsomal fraction of the rabbit liver.

Amphetamine Disappearance and Phenylacetone Formation—Supernatant fluid obtained from 20 gm. of rabbit liver was incubated at 37° for 1 hour with 100 μmoles of nicotinamide, 10 μmoles of TPN, 100 μmoles of $MgCl_2$, 120 μmoles of *l*-amphetamine, 10 ml. of phosphate buffer, pH 7 (0.2 M), and examined for the presence of phenylacetone, the deaminated metabolite of amphetamine. Apparent phenylacetone formed in the enzymatic

TABLE III

Requirement for Reduced TPN

0.3 ml. of liver microsomal fraction, 0.6 μmole of *l*-amphetamine, 5 μmoles of $MgCl_2$, 5 μmoles of nicotinamide, 0.5 ml. of 0.2 M phosphate buffer, pH 7.4, added cofactors, and water to make a final volume of 4 ml. were incubated at 37° for 2 hours.

Cofactors added	*l*-Amphetamine metabolized
	μmole
TPN 1 μmole*	0.01
" 0.1 μmole, glucose-6-phosphate 5 μmoles	0.03
" 0.1 " " 5 " glucose-6-phosphate dehydrogenase 1 mg.	0.25
TPNH 1 μmole*	0.26
DPNH 1 "	0.03
Soluble supernatant from 100 mg. of rabbit liver + TPN 0.1 μmole	0.26

* These cofactors were added in five divided portions over a period of 90 minutes.

deamination of amphetamine was extracted from the incubation mixture with carbon tetrachloride and treated with 2,4-dinitrophenylhydrazine as described under "Methods." The absorption spectra of the hydrazone of the enzymatically formed phenylacetone and that of the hydrazone of an authentic sample of phenylacetone were identical. Further evidence for the identity of the hydrazone of phenylacetone was obtained by ascending paper chromatography with water, *t*-butanol, and *n*-butanol (6:6:5) as the solvent system. The apparent and authentic hydrazones of phenylacetone were found to have the same R_F (0.70).

The quantity of phenylacetone in the enzymatic deamination of *l*-amphetamine was equivalent to only about one-half that of the *l*-amphetamine metabolized (Table IV). However, phenylacetone incubated with rabbit microsomes and TPNH was also metabolized. On incubation of 0.5, 1, and 2 μmoles of phenylacetone with rabbit liver microsomes, about

the same percentage of the phenylacetone disappeared. It was possible, therefore, to make an approximate correction for the metabolism of phenylacetone formed in the enzymatic deamination of *l*-amphetamine. The re-

TABLE IV

Enzymatic Deamination of l-Amphetamine to Phenylacetone

Each beaker containing 1.5 ml. of rabbit liver microsomal fraction, 25 μmoles of $MgCl_2$, 25 μmoles of nicotinamide, 2 ml. of phosphate buffer, pH 7.4 (0.2 M), added cofactors, substrates, and water to make a final volume of 10 ml. was incubated at 37° for 2 hours. Experiments 1 and 1A contained 3 μmoles of TPNH added in five divided portions over a period of 90 minutes. Experiments 2 and 2A contained 10 μmoles of glucose-6-phosphate, 1 mg. of glucose-6-phosphate dehydrogenase, and 0.3 μmole of TPN.

Experiment No.	Substrate added	Amphetamine metabolized	Phenylacetone found	Phenylacetone metabolized	Calculated amount of phenylacetone formed
		μmoles	*μmole*	*per cent*	*μmoles*
1	*l*-Amphetamine 4 μmoles	1.26	0.63		1.21
1A	Phenylacetone 1.0 μmole			48	
2	*l*-Amphetamine 4 μmoles	1.10	0.66		1.02
2A	Phenylacetone 1.0 μmole			35	

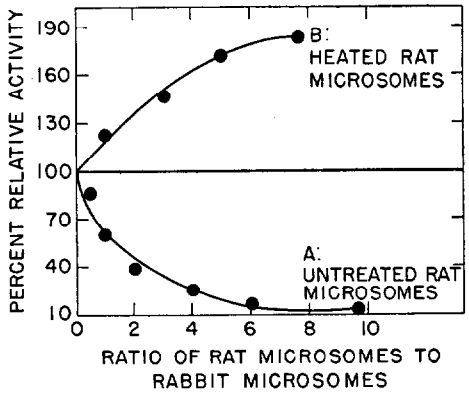


FIG. 3. Inhibition and activation of amphetamine-deaminating activity of rabbit liver microsomes by rat liver microsomes. *A*, each beaker contained 0.6 μmole of *l*-amphetamine, 0.1 μmole of TPN, 5 μmoles of $MgCl_2$, 5 μmoles of nicotinamide, 0.5 ml. of phosphate buffer, pH 7.4 (0.2 M), microsomal fraction and soluble supernatant fraction obtained from 100 mg. of rabbit liver, and varying amounts of microsomes obtained from rat liver. The reaction mixture was incubated at 37° for 2 hours. *B*, same as *A*, but rat microsomes were preheated for 2 minutes in a boiling water bath.

sults shown in Table IV indicate that about 1 mole of phenylacetone was formed for each mole of amphetamine metabolized.

From 50 to 100 per cent of the theoretical amount of ammonia was found to be liberated in the enzymatic deamination of amphetamine, but,

TABLE V

Substrate Specificity

0.3 ml. of rabbit liver supernatant fraction was incubated at 37° for 2 hours with 0.6 μmole of substrate and cofactors as described under "Methods." The reaction mixture was examined for the amount of substrate remaining. In the absence of TPN none of the substrates were metabolized.

Substrate	Relative activity
	per cent
l-Amphetamine	100
d-Amphetamine	27
l-*p*-Hydroxyamphetamine	14
l-Ephedrine	150
d-Ephedrine	11
l-Norephedrine	70
dl-Methylamphetamine	77
dl-1-Phenyl-1-aminopropane	40
dl-1-Phenyl-3-aminobutane	70
Phenylethylamine*	30
dl-α-Phenylethylamine*	27
Tyramine*	0
Benzylamine*	0
2-Aminoheptane	20
Isoamylamine*	0

* The supernatant fluid was preincubated at 37° for 10 minutes with 10^{-4} M isopropylisonicotinylhydrazine before the addition of substrates metabolized by monoamine oxidase. At this concentration isopropylisonicotinylhydrazine completely inhibits monoamine oxidase activity (10) without effecting amphetamine-deaminating activity.

owing to the relatively large amounts of endogenous ammonia also formed by the enzyme preparations, an accurate balance study could not be made.

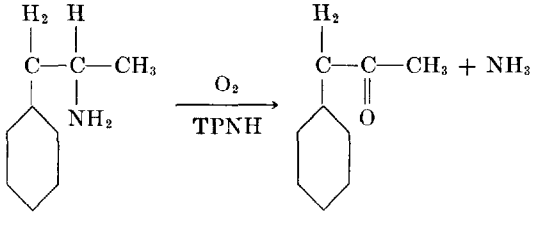
Enzyme Activity in Various Species—The activity of the amphetamine-deaminating enzyme was examined in dog, guinea pig, and rat liver supernatant fraction. Only small amounts of enzyme activity were found in these species compared to the rabbit. The possibility was entertained that the low degree of deamination activity resulted from inhibitory factors in microsomes. This was examined by measuring the effect of microsomes of dog, guinea pig, and rat liver on the enzyme activity of rabbit microsomes. The activity of the rabbit liver microsomes was markedly de-

pressed in the presence of the microsomes of these species. On the other hand, the rabbit microsomal enzyme was not inhibited by the soluble supernatant fractions of dog, guinea pig, or rat liver. These observations indicated that an inhibitory factor was present in the microsomes of dog, guinea pig, and rat liver. Fig. 3, *A* shows the inhibitory action of rat microsomes on the amphetamine-deaminating activity of rabbit liver microsomes. When the microsomal fraction of rat liver was heated at 100° for 2 minutes prior to its addition to the rabbit enzyme, a marked stimulation of enzyme activity was observed (Fig. 3, *B*). These results indicate the presence of a heat-stable activating factor which is masked by a heat-labile inhibitory factor. Preheated microsomes of dog, guinea pig, and rabbit liver also possessed stimulatory activity.

Substrate Specificity—The metabolism of a number of amines by rabbit liver supernatant fraction is recorded in Table V. Substrates having a phenylpropylamine or phenylbutylamine structure were extensively metabolized and the enzyme preparation showed relative specificity for the levo isomers. None of the amines were metabolized in the absence of TPN. It appeared that phenolic-substituted amines, phenylethylamines, and aliphatic amines were metabolized slightly or not at all. It was possible that the arylamines could also be metabolized by hydroxylation of the aromatic nucleus. However, it seemed unlikely that the phenylisopropylamines were hydroxylated in the rabbit, since it was demonstrated that this species could not hydroxylate these amines *in vivo* (2, 5). It seemed likely that the rabbit liver microsomal enzyme system metabolized the amphetamine analogues by deamination.

DISCUSSION

On the basis of the studies described in this paper, the over-all reaction for the enzymatic deamination of amphetamine is represented in the accompanying scheme. Amphetamine is deaminated to phenylacetone and ammonia in the presence of reduced TPN and oxygen by an enzyme system which is localized in the microsomal fraction of the rabbit liver. A reservoir of reduced coenzyme is maintained by TPN-dependent dehydrogenases and their oxidizable substrates present in the soluble supernatant fraction.



The enzyme which deaminates amphetamine differs from other deaminating enzymes such as monoamine oxidase, D-amino acid oxidase, L-amino acid oxidase, and glutamic acid dehydrogenase with respect to its substrate specificity, cellular localization, and cofactor requirements.

The rôle of reduced TPN in an enzyme system catalyzing the oxidative deamination of an amine is not understood. Reduced TPN could conceivably act by the generation of hydrogen peroxide through the transfer of its hydrogen by an intermediate electron transport system to molecular oxygen. Preliminary studies indicate that hydrogen peroxide generated from D-amino acid oxidase was unable to replace TPNH in the deamination of amphetamine by rabbit liver microsomes. The activating action of heated rat microsomes suggests the requirement for other factors besides TPNH. The nature of the additional cofactor is unknown.

Previous studies have shown considerable species differences in the metabolism of amphetamine *in vivo* (2). The dog and the rat transformed amphetamine mainly by hydroxylation, while the rabbit metabolized the drug, presumably by deamination. The species differences in the metabolism of amphetamine may be explained in part by the presence of inhibitory factors in the dog and rat. Inhibitory factors in these species may act by blocking the deamination of amphetamine so that the drug is excreted unchanged or metabolized by alternative pathways involving hydroxylation.

It is becoming increasingly evident that enzymes in liver microsomes which have a specific requirement for reduced TPN and oxygen are of major importance in the detoxification of many drugs and foreign organic compounds. Such enzyme systems have been found to carry out a variety of reactions such as dealkylation of alkylamines (11, 12), side chain oxidation of barbiturates,[1] cleavage of aromatic ethers,[2] and hydroxylation of aromatic compounds (13). Another property common to these enzyme systems is their inhibition by β-diethylaminoethyl diphenylpropylacetate (14, 15).

SUMMARY

An enzyme system in rabbit liver microsomes catalyzes the deamination of amphetamine to yield phenylacetone and ammonia. The enzyme system requires reduced triphosphopyridine nucleotide and oxygen. The TPN-dependent dehydrogenases in the soluble supernatant fraction of the liver serve to maintain a reservoir of reduced triphosphopyridine nucleotide.

Species differences in the metabolism of amphetamine may be explained, in part, by the presence of inhibitory factors in the microsomes of the dog

[1] Cooper, J. R., and Brodie, B. B., unpublished findings.

[2] Axelrod, J., unpublished work.

and rat liver. A heat-stable factor which can stimulate the enzymatic deamination of amphetamine is also present in the microsomes.

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